Summary:

In the current scenario, the pharmaceutical companies are investing in Research and Development (R&D) to develop and introduce new chemical entities into the market. Pharmaceutical industry is growing day by day at a faster pace. The increasing concern over the R&D sector in the context of world trade organization (WTO), global patent systems forced the pharmaceutical industry to concentrate more on R&D activities. New technology, new techniques and sophisticated instruments increased the scope of the research activity further.

Identification and quantification of impurities is a crucial task in pharmaceutical process development for quality and safety. Related components in pharmaceuticals are the impurities which are unwanted chemicals that remain with the active pharmaceutical ingredients (APIs), or develop during stability testing, or develop during formulation or upon aging of both API and formulated APIs to medicines. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Varied analytical methodologies were employed for the determination of related components in pharmaceuticals. There is a great need for development of analytical methods for new emerging drugs. The developed methods for estimation of impurities should be stability indicating. As per the current ICH requirements the evaluation of stability sample must be carried out using stability indicating analytical methods. A review of literature reveals a

large number of methods reported over a period of 3-4 decades under the nomenclature of "Stability-indicating". How ever most of the reported methods fall short in meeting the current regulatory requirement.

Six different Active Pharmaceutical Ingredients (API's) and five of their dosage forms were selected for research work, keeping objective to develop novel HPLC methods for the determination of related components. Novel LC methods were developed to determine the related components in different classes of pharmaceutical compounds which includes Nateglinide (Oral glucose regulator), Ranolazine (Anti anginal), Brimonidine Tartrate (treatment of open-angle glaucoma), Tolterodine tartrate (treatment of urinary urge incontinence and other symptoms related to unstable bladder), Zafirlukast (treatment of asthma) and Tadalafil (Enhances erectile function). The literature survey reveals that there is no stability indicating liquid chromatographic methods for estimation of related components in Nateglinide, Ranolazine, Brimonidine tartrate, Tolterodine tartrate. Moreover literature survey reveals the absence of methods for enantiomeric purity determination of Nateglinide, separation of meta and para isomers of Zafirlukast from Zafirlukast, a method for separation of (R, R)- Tadalafil from its enantiomer. Literature references are available for some other purpose of estimating the drug substance in human plasma, urine etc. Suitable novel stability indicating analytical methods were developed, keeping the current regulatory requirements in mind and the developed methods were extensively validated. The performance of the

developed novel LC methods for related components estimation has been verified by applying the same to evaluate the quality of bulk samples of API during its stability studies. The developed LC methods are performed well for the quality evaluation of stability samples.

Chapter 1: Importance of Development and validation of analytical methods for the determination of related components in pharmaceutical compounds using chromatography technique.

In this chapter a brief outline on importance of development of novel liquid chromatographic methods for related components estimation in pharmaceutical research and development, Quality guide lines and ICH methodology for stability indicating method development were explained.

Chapter 2: This chapter was divided into part-A and part-B.

Part-A deals with development and validation of a novel LC method for determination of related components in Nateglinide and part-B deals with a validated reverse phase chiral liquid chromatographic method for the enantiomeric purity determination of Nateglinide in bulk drug samples and pharmaceutical dosage forms.

Nateglinide is N-(trans-4-isopropylcyclohexyl-carbonyl)-D-phenylalanine is novel oral glucose regulator, which was recently approved for the treatment of type 2 diabetes mellitus. Starlix® (Nateglinide) increases insulin release from the pancreatic ß-cells through inhibition of ATP-dependent potassium channels.

A novel isocratic reverse phase liquid chromatographic (RP-LC) assay method has been developed for the quantitative determination of Nateglinide and its related components namely impurity-1 and impurity-2 in bulk drug and in pharmaceutical dosage form, used for the treatment of type II diabetes mellitus. The developed method is stability indicating and also can be used for stability testing. The chromatographic separation was achieved on C-8, (150*4.6) mm, 3.5 µm particle size stationary phase. The liquid chromatographic method employs solution A as mobile phase. Solution A contains a mixture of phosphate buffer pH 3.0: acetonitrile (50:50 *v/v*). The flow rate was 1.0 mL min^{-1} and the detection wavelength was 210 nm (UV detection technique). In the developed liquid chromatographic method the resolution between Nateglinide and its potential impurities namely impurity-1 and impurity-2 was found to be greater than 5.0. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. Considerable degradation was found to occur in acid medium, alkaline medium and oxidative stress conditions. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 99.2%. The developed chromatographic method was validated with respect to linearity, accuracy, precision and robustness.

Part-B: A validated reverse phase chiral liquid chromatographic method for the enantiomeric purity determination of Nateglinide in bulk drug samples and pharmaceutical dosage forms.

A simple isocratic reverse phase chiral HPLC method was developed for the enantiomeric purity determination of Nateglinide in bulk drugs and dosage forms with a short run time of about 20min. Chromatographic separation of Nateglinide and its enantiomer was achieved on a bonded macro cyclic glycopeptides (Chirobiotic-T column (250 x 4.6)mm with 5µm particle size) stationary phase. Bonded macrocyclic glycopeptides stationary phase found to be enantioselective for L and D enantiomers of Nateglinide with a resolution (R_S) of greater than 2.8. The mobile phase used was a mixture of buffer and acetonitrile in the ratio of 70: 30 (*v/v*). Buffer consists of 5mMof Tetra-n-butyl ammonium hydrogen sulfate, pH adjusted to 3.5 using diluted ammonia solutions (1 in 10). The test concentration is 1.0 mg mL^{-1} in diluent (6:4 (*v/v*) acetonitrile and water). The developed method is capable of detecting the L-Nateglinide at a level of 0.07µg with respect to test concentration of 1000µg mL^{-1} for a 10µL injection volume. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness. The percentage recovery of L-Nateglinide in bulk drug samples and in dosage forms ranged from 97.6 to 101.6%. The test solution was found to be stable in the diluent for 48h after the preparation. The prepared mobile phase is also stable for a test period of 48h.

Chapter 3: Development and validation of a new analytical method for the determination of related components and assay of Ranolazine in bulk drug and pharmaceutical dosage forms by LC.

Ranolazine is an Anti -anginal and anti-ischemic agent. Ranolazine is designated chemically as (RS)-N-(2, 6-Dimethyl phenyl)-2-{4-[2-hydroxy-3-(2-methoxy-phenoxy)-propyl]-piperazin-1-yl}-acetamide. Ranexa (Ranolazine) is available as an extended-release tablet for oral administration; it has a cardio protective effect against ischemia reperfusion injury, without effecting hemodynamics, both in vitro and in vivo.

A novel liquid chromatographic method has been developed and validated for the determination of Ranolazine, its potential four impurities in drug substance and drug products. Efficient chromatographic separation was achieved on a C18 stationary phase (150 x 4.6 mm, 3.0 microns particles) with simple mobile phase combination delivered in gradient mode at a flow rate of 1.0 mL min^{-1} at 210 nm. In the developed method, the resolution between Ranolazine and its four potential impurities was found to be greater than 2.0. Regression analysis shows a correlation coefficient value greater than 0.999 for Ranolazine and for its four impurities. This method was capable to detect all four impurities of Ranolazine at a level below 0.002 μgmL^{-1} with respect to test concentration of 1.0 mg mL^{-1} for a 10 µL injection volume. The method has shown good, consistent recoveries for Ranolazine (99.2-101.5%) and for its four

impurities (97.2-101.5). The test solution was found to be stable in the diluent for 48 h. The drug was subjected to stress conditions. The mass balance was found close to 99.5%.

Chapter 4: Development and validation of a new LC method for analysis of Brimonidine Tartrate and related compounds.

Brimonidine tartrate is designated chemically as 5-bromo-6-(2-imidazolidinylideneamino) quinoxaline L-tartrate. Brimonidine tartrate is a potent α_2 - adrenoceptor agonist which decreases intra ocular pressure (IOP) in humans suffering with open-angle glaucoma or ocular hypertension.

A novel liquid chromatographic method for analysis three potential impurities in Brimonidine tartrate drug substance has been developed and validated. Efficient chromatographic separation was achieved on a C8 (250* 4.6) mm, 5µm particles column with a simple mobile-phase gradient at a flow rate of 1.0 mL min^{-1}. Quantification was achieved by use of ultraviolet detection at 248 nm. Resolution between Brimonidine tartrate and its three potential impurities was greater than 3.0. Regression analysis showed a correlation coefficient value was greater than 0.999 for Brimonidine and its three impurities. The method was capable of detecting all three impurities of Brimonidine tartrate at levels below 31.0 µg mL^{-1} in a test concentration of Brimonidine tartrate of 1.0 mg mL^{-1} and for an injection volume of 10 µL. A solution of Brimonidine tartrate in acetonitrile-water 2:8 (*v/v*) was stable for 48h. The drug was subjected to

stress conditions as prescribed by the ICH. Degradation was found to occur slightly under oxidative stress conditions but the drug was stable to aqueous, acidic, basic hydrolysis, photolytic and thermal stress. The assay of the stressed samples was calculated relative to a qualified reference standard and the mass balance was found close to 99.8%. The method was validated for linearity, accuracy, precision and robustness.

Chapter 5: Development and validation of a new analytical method for the determination of related components in Tolterodine Tartarate using LC.

Tolterodine ((R)-N, N-diisopropyl-3-(2-hydroxy-5-methylphenyl)-3-phenylpropanamine) is a new muscarinic receptor antagonist intended for the treatment of urinary urge incontinence and other symptoms related to unstable bladder. DETROL ™ is a generic name for Tolterodine tartarate tablets.

A novel liquid chromatographic method has been developed and validated for the determination of Tolterodine tartarate, for its potential three impurities in drug substances and drug products. Efficient chromatographic separation was achieved on a C8 stationary phase (150 x 4.6 mm, 3.5 µm particles) with a simple mobile phase combination delivered in an isocratic mode at a flow rate of 0.8 mL min^{-1} and quantitation was carried out using ultraviolet detection at 210nm. Microwave assisted degradation procedure was employed for stress testing studies in addition to the conventional way of a refluxing method. The

results of both studies were compared. In the developed LC method, the resolution between Tolterodine and its three potential impurities was found to be greater than 3.0. Regression analysis shows a correlation coefficient value greater than 0.999 for Tolterodine and for its three impurities. The developed method was capable to detect all three impurities of Tolterodine at a level below 0.004 μgmL^{-1} with respect to a test concentration of 0.5 mg mL^{-1} for a 10 µL injection volume. The precisions for all three impurities and for Tolterodine were found to be within 1.0 RSD% at its specification level. The method has shown good, consistent recoveries for Tolterodine (98.3-100.7%) and for its three impurities (94.5-103.0%). The test solution was found to be stable in the diluent for 48h. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation as prescribed by ICH. Degradation was found to occur in alkaline stress condition, while the drug was stable to water hydrolysis, acid hydrolysis, oxidative stress, photolytic and thermal stress. The assay of stressed samples was calculated against a qualified reference standard and the mass balance was found close to 99.4%. Microwave degradations were very fast and comparable to the conventional way of the refluxing method. Robustness studies were carried out and suggested that system suitability parameters were unaffected by small changes in critical factors. The validated method was successfully applied for the determination of Tolterodine tartarate and its related three impurities in drug substances and drug products.

Chapter 6: LC separation of para and meta isomers of Zafirlukast in bulk drug samples and pharmaceutical dosage forms using a chiral stationary phase.

Zafirlukast (Accolate®) {3-[2-Methoxy-4-(toluene-2- sulfonyl -aminocarbo Nylaminocarbonyl)-benzyl] 1-methyl-1H-indol-5-yl}-carbamic acid cycloPentyl ester is an oral leukotriene receptor antagonist (LTRA), selective and competitive orally administered inhibitor of the cysteinyl leukotrienes for the maintenance treatment of asthma.

A simple, rapid and sensitive high performance liquid chromatographic method was developed for the separation and quantification of positional isomers of Zafirlukast in bulk drugs and dosage forms using a chiral column. Elution time was 20 min in normal phase mode and ultra violet detection was carried out at 240 nm. Efficient separation was achieved on an immobilized amylose-based Chiralpak-IA column using n-hexane/ethanol/ trifluoroacetic acid/diethyl amine (65:35:0.1:0.1, *v/v*) as the mobile phase. Resolutions between ortho, meta and para isomers of Zafirlukast were found to be greater than 2.9. The active pharmaceutical ingredient was extracted from tablets using tetrahydrofuran. The calibration graphs for meta and para isomers of Zafirlukast were linear ($r > 0.999$) when ranging from the limit of quantitation to 200% of specification limit. The method showed excellent recoveries for both Zafirlukast isomers identified in bulk and formulated products. The test

solution was found to be stable in the diluent (mobile phase is the diluent) for 48h after preparation. The developed LC method was validated with respect to linearity, accuracy, precision and robustness.

Chapter 7: Chiral separation of (*R, R*)-tadalafil and its enantiomer in bulk drug samples and pharmaceutical dosage forms by chiral RP-LC.

Tadalafil is designated chemically as (*6R, 12aR*)-2,3,6,7,12,12*a*-hexahydro-2-methyl-6-(3,4-methylene dioxyphenyl) pyrazino (1′, 2′: 1,6) pyrido- (3,4-b) indole-1, 4-dione. Cialis is a generic name for Tadalafil. Tadalafil is a selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type-5 (PDE-5). Through the inhibition on PDE-5, Tadalafil increases the concentration of cyclic guanosine monophosphate (cGMP), producing smooth muscle relaxation and increased blood flow to the corpus cavernosum, thereby enhancing erectile response following appropriate sexual stimulation.

A new simple isocratic chiral RP-LC method has been developed for the separation and quantification of the enantiomer of (*R,R*)-tadalafil in bulk drugs and dosage forms with an elution time of about 20 min. Chromatographic separation of (*R,R*)-tadalafil and its enantiomer was achieved on a bonded macro cyclic glycopeptide stationary phase. The method resolves the (*R, R*)-tadalafil and its enantiomer with a resolution (Rs) greater than 2.2 in the developed chiral RP-LC. The test concentration is 0.4 mg mL^{-1} in the mobile phase. This method is capable of detecting the enantiomer of (*R,R*)-tadalafil up to 0.013 μgmL^{-1} with respect to test

concentration 400 μg mL^{-1} for a 20 μL injection volume. The developed chiral RP-LC method was validated with respect to linearity, accuracy, precision and robustness. The percentage recovery for the enantiomer of (*R,R*)-tadalafil in bulk drug samples and in dosage forms ranged from 97.8 to 102.5%. The test solution was found to be stable in the mobile phase for 48h after preparation.

Table 8.1 Summary of analytical methods developed for six pharmaceutical compounds, developed methods linearity range, precision values and journal published.

Drug Name	Method Developed	Linearity range and Precision	Journal Published
Nateglinide	<u>For Related components determination :</u> Column: Zorbax C8, (150 x 4.6) mm, 3.5µm particle size. Mobile phase: 10mM NaH2PO4.1H2O, pH adjusted to 3.0 using H3PO4 and acetonitrile in 50:50 (*v/v*) ratio. Column temperature : 25 ± 2°C Wavelength of detection : 210 nm Injection volume : 10µL Diluent : Acetonitrile Flow rate : 1.0 mL min^{-1} Run time : 30 minutes. Retention of Nateglinide is ~ 7 min Relative retention of Impurit-1 is ~0.74 and for Impurity-2 is ~2.22.	LOQ to 200% concentration with respect to impurity specification. Precision (RSD%) for Assay and related components at specification level is < 2.0	Journal of Chromatographia
	<u>For Enantiomeric purity estimation of Nateglinide:</u> Column: Chirobiotic-T, (250x4.6) mm with 5µm particle size. Mobile phase: Buffer:Acetonitrile:70:30(*v/v*) Flow rate : 1.0 mL min^{-1} Column temperature: 25 ± 2°C Wavelength :210 nm Injection volume : 10µL Run time : 30 min Diluent: Acetonitrile: water (6:4, *v/v*) Buffer: 5 mM of tetra-n-butyl ammonium hydrogen sulfate, pH adjusted to 3.5 using diluted ammonia solution (1 in 10mL). Retention of Nateglinide is about 8.8 min Relative retention of L-Nateglinide is 0.79	LOQ to 200% concentration with respect to impurity specification. Precision (RSD%) for Assay and related components at specification level < 0.1	Journal of Analytical Chemistry
Ranolazine	Column: Inertsil ODS (150 x 4.0) mm with 3.0 µm particle size. Elution: Gradient. Mobile phase-A: Buffer: acetonitrile: 90:10(*v/v*) Mobile phase-B : Acetonitrile: water:90: 10(*v/v)* Flow rate : 1.0 mL min^{-1} Column temperature : 40° Run time : 30 min Wavelength: 210 nm Injection volume : 10µL Diluent: Mobile phase-A and mobile phase-B in 1:1(*v/v*) ratio. Gradient Program: Time (min)/ % solution B: 0.01/0, 5/0, 10/20, 20/50, 25/50, 27/0 and 30/0 Buffer: 0.02M sodium dihydrogen phosphate and 2 mL of Triethylamine, pH adjusted to 4.5 using diluted phosphoric acid (1mL in 10 mL of Milli-Q water). Retention time: Ranolazine ~12 min. Relative Retention Time: Impurity-1 - ~ 0.39	LOQ to 200% concentration with respect to impurity specification. Precision (RSD%) for Assay and related components at specification level < 1.9	Journal of Chromatographia

	Impurity-2 - ~ 0.96 Impurity-3 - ~ 1.12 Impurity-4 - ~1.35		

Drug Name	Method Developed	Linearity range and Precision	Journal Published
Brimonidine tartrate	Column: Inertsil C-8, (150 x 4.6) mm, 3.5µm particle size Mobile phase: Solution-A: 0.01 M sodium dihydrogen Phosphate monohydrate and 0.02 M n-Hexane sulfonic acid sodium salt, pH adjusted to 3.0 using diluted phosphoric acid (1mL in 10 mL of Milli-Q water). Solution-B: Acetonitrile: methanol (50:50 *v/v*). Gradient program Time (min) / % solution B: 0/20, 10/20, 35/70, 37/20 and 40/20. Flow rate : 1.0 mL min^{-1} Run time : 40 min Column temperature : 25 ± 2°C Wavelength of detection : 248 nm Injection volume : 10µL Diluent : Water: acetonitrile (8:2, *v/v*) Retention time: Brimonidine tartrate~17.7 min Relative Retention Time:Impurity-1 ~ 0.41 Impurity-2 ~ 0.74 Impurity-3 ~ 0.86	LOQ to 200% concentration with respect to impurity specification. Precision (RSD%) for Assay and related components at specification level < 1.0	Journal of Chromatographia
Tolterodine tartrate	Column: Zorbax SB C8, (150x 4.6) mm, 3.5µm particle size Mobile phase: Buffer: acetonitrile: 60:40 (*v/v*) Flow rate : 0.8 mL min^{-1} Column temperature : 27 ± 2°C Wavelength of detection : 210 nm Injection volume: 10µL Run time : 30 min Diluent : Acetonitrile: water (1:1, *v/v*) Buffer: 50mM sodium dihydrogen phosphate monohydrate and 5 mL of triethylamine, pH adjusted to 2.5 using diluted phosphoric acid (1mL in 10 mL of Milli-Q water). Retention time of Tolterodine peak: about 4.9 Relative Retention Time : Impurity-1 ~0.41 Impurity-2 ~ 0.74 Impurity-3 ~ 0.86	LOQ to 200% concentration with respect to impurity specification. Precision (RSD%) for Assay and related components at specification level < 1.0	Journal of Chromatographia
Zafirlukast	Column: chiral pack IA (250x4.6) mm with 5µm particle size. Mobile phase: n-hexane, ethanol, Trifluoro acetic acid (TFA)and diethyl amine (DEA) in the ratio of 65:35:0.1:0.1 (*v/v/v/v*). Flow rate : 1.0 mL min^{-1} Column temperature : 27°C Wavelength of detection : 240 nm Injection volume : 10µL Run time : 30 min Diluent: Tetrahydrofuran and mobile phase	LOQ to 200% concentration with respect to impurity specification. Precision (RSD%) for meta and para isomers Zafirlukast at	Journal of Chromatographia

	(3:7, *v/v*) Retention time of Zafirlukast peak : about 12.1 min Relative Retention Time of meta isomer of Zafirlukast peak: about 1.18 Relative Retention Time of meta isomer of Zafirlukast peak: about 1.40	specification level < 0.9	

Drug Name	Method Developed	Linearity range and Precision	Journal Published
Tadalafil	Column: Chirobiotic-T, (250x4.6) mm with 5µm particle size. Mobile phase: Water, methanol and acetonitrile (55:40:5) (*v/v/v*). Flow rate: 1.0 mL min^{-1} Run time : 30 min Column temperature : 27 °C Wavelength of detection : 220 nm Injection volume : 20µL Diluent : Mobile Phase Retention time of (R,R) - tadalafil peak : About 12.7 min Relative Retention Time of enantiomer of (R,R) – tadalafil : About 0.84	LOQ to 200% concentration with respect to impurity specification. Precision (RSD%) for % enantiomer of (*R, R*)-tadalafil at specification level < 0.9	Journal of Chromatographia